Bioorganic & Medicinal Chemistry Letters 28 (2018) 134–139

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Small molecule inhibitors of anthrax edema factor

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ARTICLE INFO

Article history:
Received 31 August 2017
Revised 23 November 2017
Accepted 24 November 2017
Available online 24 November 2017

Keywords:
Anthrax
Edema factor
Covalent inhibitor

ABSTRACT

Anthrax is a highly lethal disease caused by the Gram-(+) bacteria *Bacillus anthracis*. Edema toxin (ET) is a major contributor to the pathogenesis of disease in humans exposed to *B. anthracis*. ET is a bipartite toxin composed of two proteins secreted by the vegetative bacteria, edema factor (EF) and protective antigen (PA). Our work towards identifying a small molecule inhibitor of anthrax edema factor is the subject of this letter. First we demonstrate that the small molecule probe 5′-Fluorosulfonylbenzoyl 5′-adenosine (FSBA) reacts irreversibly with EF and blocks enzymatic activity. We then show that the adenosine portion of FSBA can be replaced to provide more drug-like molecules which are up to 1000-fold more potent against EF relative to FSBA, display low cross reactivity when tested against a panel of kinases, and are nanomolar inhibitors of EF in a cell-based assay of cAMP production.

Anthrax results from infection by *Bacillus anthracis* and is highly lethal unless quickly diagnosed and treated. *B. anthracis* makes two bipartite toxins generated by the combination of three proteins secreted by the bacteria. Edema Toxin (ET) composed of edema factor (EF), an adenylate cyclase,[1] plus protective antigen (PA), and Lethal Toxin (LT), made of lethal factor (LF), a zinc-dependent metalloproteinase,[2] plus PA, are considered major contributors to the pathogenesis and death of humans exposed to *B. anthracis*.[3]

At present, antibiotics and antibody therapies are available to prevent the onset of disease following exposure; however, once a patient is symptomatic these agents rapidly lose their effectiveness. This is due to the release of ET and LT immediately following germination,[4] and the rapid uptake of these toxins by cell surface receptors which are ubiquitous throughout the host. Once inside cells, EF and LF are no longer accessible to antibodies and initially act to promote the infection by disarming the body's immune system.[5] Both EF and LF target cell signal pathways resulting in abnormal cell function and cell death.[6] Recent studies using mice modified for expression of anthrax toxin receptors have demonstrated that lethality from exposure to ET and LT results from each toxin targeting specific tissues of the host.[7] In the case of ET, hepatocytes appear to be the major target, whereas LT primarily induces lethality through its effects on cardiomyocytes and vascular smooth muscle cells. Cardiovascular system damage has been linked to the intracellular activity of LF in the rabbit model[8] while EF is the cause of massive edema seen in some human patients infected by *B. anthracis*.[9] Recent *ex vivo* studies using a rat aortic ring model implicate ET as a contributor to shock in the host due to arterial relaxation.[10] Due to their unique ability to evade normal clearance mechanisms of the host,[11] both toxins can contribute to mortality and morbidity of human patients long after the bacteria have been cleared from the blood stream by antibiotics.[9,12]

Competitive[13] and allosteric[14] inhibitors of EF have been reported. However, these compounds display low affinity, or are derivatives of ATP[15] which exhibit poor drug-like properties making them unlikely candidates for therapeutic intervention to treat anthrax in humans. To address this unmet need, we present below our initial studies towards identifying potent small molecule inhibitors of anthrax EF.

The lack of an FDA approved small molecule drug to treat EF intoxication results in part from the challenging nature of the problem to be solved. In addition to the need for specificity, a second consideration is the very high turnover rate ($k_{cat} \sim 1500\ s^{-1}$) exhibited by this enzyme.[16] A third issue is the very high intracellular concentrations[17] (~5 mM) of its substrate, ATP, which presents a significant challenge for any competitive inhibitor.

One solution to these problems is to employ a covalent edema factor inhibitor (EFI) to irreversibly silence the enzyme. Covalent inhibitors appear well suited for the treatment of acute diseases and may be used at lower doses due to their higher efficiency and lower need for optimized PK parameters.[18–20] This approach also addresses the problem associated with active toxin remaining after antibiotic treatment since any circulating and intracellular EF

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https://doi.org/10.1016/j.bmcl.2017.11.040
0960-894X/

would be eliminated after reacting with a covalent EFI. Typical covalent inhibitors targeting kinases[21] are composed of a recognition element for the ATP site combined with an electrophilic group that is reactive towards a cysteine residue. Although no cysteine residues are located near the ATP binding site of EF, an X-ray crystal structure of 3′-deoxy-ATP bound to EF[22] does show three lysine residues in contact with the triphosphate group attached to the adenosine core structure (Fig. 1). This observation suggests that all three terminal amino groups have the potential to react as nucleophiles with an electrophilic group if correctly positioned in the ATP binding site of EF.

Although not as common as cysteines, lysine residues are capable of acting as nucleophiles and deactivating enzymes by covalent bond formation to small molecules substrates.[23–27]

After surveying the known small molecule inhibitors which react with lysine residues, we selected the affinity label 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) as a model compound for proof of concept studies (Fig. 2). Being a derivative of adenosine, this compound appeared ideally suited to test the hypothesis that EF could be inactivated by a small molecule covalently modifying the ATP binding site. Examination of the protein crystal structure of 3′deoxy-ATP bound to EF[22] indicated that intermolecular hydrogen bonds between the adenine ring and Thr548 provided a *recognition element* for binding to the ATP site while the ribose portion of adenosine displayed only minimal interaction and appeared to act simply as a *linking group* to the triphosphate tail. This data combined with molecular modeling (Fig. S7) provided the initial support that FSBA could indeed bind in a similar manner to EF with the fluorosulphonyl group capable of acting as the *electrophilic group* towards any of the three lysine terminal amino groups.

To test this hypothesis, we conducted concentration-time dependent studies[28] with FSBA. As shown in Fig. 3A, FSBA was indeed capable of inactivating EF over time in a dose-dependent manner. Further, a plot of [FSBA] versus k_{obs} afforded a curved line (Fig. 3B) displaying saturation kinetics which supports a two-step mechanism of EF inactivation where the initial binding of FSBA by the ATP site is followed by covalent bond formation in a second step.[29]

To demonstrate that inactivation of EF by FSBA was irreversible, the enzyme was incubated with two concentrations of FSBA for 90 min followed by filtration of each sample through a size exclusion column to separate the protein from the unbound FSBA. An aliquot of each filtrate was checked for enzyme activity and compared to a control sample containing EF alone. EF exposed to FSBA resulted in decreased enzymatic activity in a dose dependent manner consistent with covalent (irreversible) binding to the enzyme (Fig. 3C).

After demonstrating that EF could be inactivated in a time-dependent manner, we began our search for novel inhibitors of EF by using FSBA as a starting point for compound synthesis. An immediate goal was to identify possible replacements for the adenosine moiety (recognition element + linking group) of FSBA while retaining the phenylsulfonyl fluoride as the electrophile given the demonstrated ability of this functional group to inactivate EF. Our strategy to divide the molecule into three regions based on these functions (Fig. 4) allowed us to design a modular synthesis of new EFIs and expedite preparation of a set of diverse analogs (Figs. S1–S6).

Kinetic inactivation data[30] for analogs based on a bicyclic recognition element is shown in Table 1, and for monocyclic analogs, in Table 2. It is important to note that when comparing the relative potency of covalent inhibitors, the recommended parameter to consider is the ratio k_{inact}/K_I, which unlike IC_{50} values, is a second order rate constant independent of pre-incubation time and substrate concentration.[29] The data in Tables 1 and 2 are ordered by increasing inhibitor potency relative to FSBA and include physico-

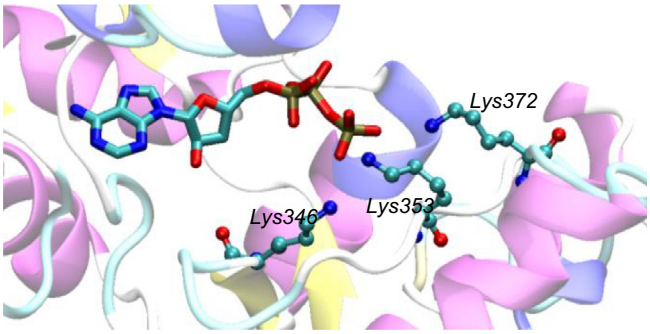

Fig. 1. The EF-3′deoxy-ATP complex showing the three lysines present in the EF ATP binding site.

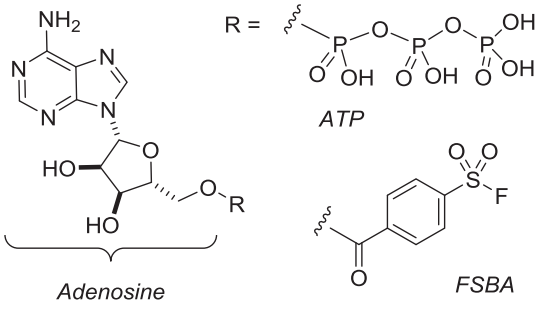

Fig. 2. Structures of ATP and FSBA.

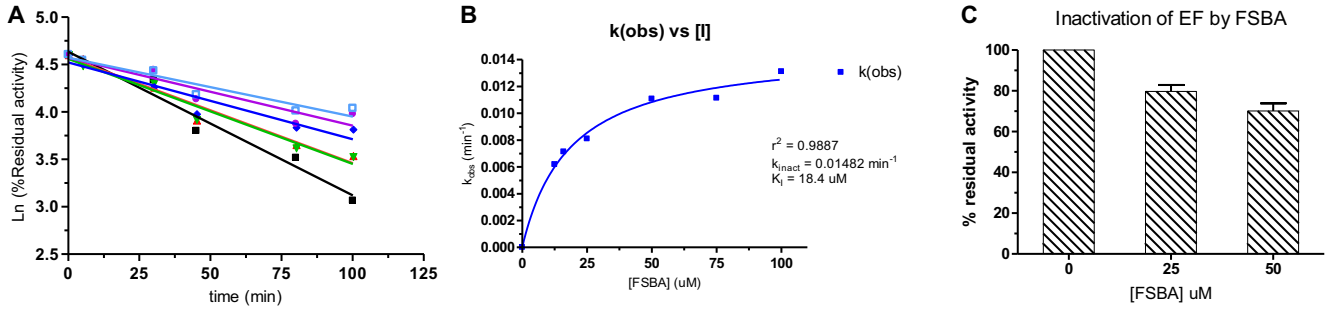

Fig. 3. A, Plot of Ln (% residual EF activity) vs. time; the slope of each line provides k_{obs}. Time dependent inactivation of EF by FSBA at various concentrations: ■: 100 μM; ▲: 75 μM; ▼: 50 μM; ◆: 25 μM; ●: 16 μM; □: 12.5 μM; B, Plot of [FSBA] versus k_{obs} where $k_{obs} = (k_{inact} \cdot [FSBA])/(K_I + [FSBA])$ and provides for the determination of k_{inact} = 0.00148 min^{-1} (SD = 0.00069) and a K_I = 18.35 μM (SD = 2.74); C, Dose dependent inactivation (n = 3) of EF by FSBA. Incubation of EF + FSBA for 90 min was followed by filtration (size exclusion) and assay for pyrophosphate production.